

Comparison of 3T3 fibroblasts growth on alginate and polyasparagine (PAA) scaffolds in mouse model

Tomasz Drewa¹, Miłosz Jasiński¹, Jolanta Polaczek², Jan Pielichowski², Celestyna Kierzenkowska-Mila¹, Joanna Łysik-Miśkowska^{3,4}, Agnieszka Krawczyk¹

¹Department of Tissue Engineering, Chair of Medical Biology, Nicolaus Copernicus University, Bydgoszcz, Poland

²Chair of Chemistry and Technology of Polymers, University of Technology, Cracow, Poland

³Chair and Department of Oncology and Brachytherapy, Nicolaus Copernicus University, Bydgoszcz, Poland

⁴Department of Pathology, Oncology Center, Bydgoszcz, Poland

Summary

Introduction. The aim of this study was to evaluate polyasparagine (PAA), a new, promising scaffold. PAA was compared with alginate, which is used in cell transplantations and may be regarded as a standard.

Materials and Methods. *In vitro* cell viability on both scaffolds was assessed. C57B1 mice were injected s. c. alginate or PAA with or without 3T3 cells. After two months specimens from sites of injection were examined and blood samples were taken for enzymatic activity estimation. Cathepsin D activity and α_1 -antitrypsin levels were measured.

Results. *In vitro* cell viability was lowest on PAA and highest in control group. Increase in levels of enzymes measured was observed in response to PAA and alginate and was lower in case of polymer seeded with cells. Increase in α_1 -antitrypsin levels was lower in case of PAA in comparison to alginate. Scaffold degradation in histopathological specimens was visible.

Conclusion The results indicate that PAA implants undergo biodegradation and nonspecific inflammatory response is comparable to alginate.

Key words: 3T3, cell culture, alginate, polyasparagic acid, tissue engineering

INTRODUCTION

Cell survival rate after transplantation and therefore its curative effect strongly depends on physical properties of the scaffold used. This material should have the appropriate durability and resilience, strongly adhere to the tissue in its deposition site and do not migrate from it. It should have remodelling properties *in vivo*. Every transplanted scaffold induces nonspecific inflammatory response, which also has an influence on cell survival [1]. Polyasparagic acid (PAA) is a new promising scaffold used experimentally in cell culture and transplantation [2]. The reason for choosing PAA for this research was the fact that there are few reports relating PAA as a scaffold [3].

It is possible that this polymer will have some application in future. The aim of this study was to examine proprieties of PAA *in vitro* and *in vivo*. It was compared with alginate, which is traditionally used as a scaffold for transplanted cells.

MATERIALS AND METHODS

Mouse fibroblasts 3T3 were cultured in 25 cm² culture flasks (Greiner, Germany), containing Dulbecco's Modified Eagle's Medium (Sigma, Germany) with 10% foetal bovine serum, at 37° C, with 5% CO₂. Fibroblasts were passaged after covering 70-80% of culture surface using 0.1% trypsin solution with 0.02% EDTA (Sigma, Germany).

In vitro assay

Cyclic PAA structure was obtained by thermal polymerization of asparagic acid under microwave irradiation. Application of microwave irradiation has permitted to reduce polymerization time to several minutes, increase the efficiency and eliminate the catalyst.

Polycondensation of cyclic PAA was performed in temperature from 170⁰C to 230⁰C, in carbonate propylene without catalyst. Phosphoric acid was used as solvent [4]. Culture flasks were covered with PAA in a following manner: 0.5g PAA was dissolved in 5ml 100% DMSO (the vial was heated and stirred until PAA was dissolved, then it was left to cool), each Petri dish was covered with approx. 1mm layer of this solution. The excess was pipetted off then they were left to dry for a week. 1% low viscosity alginate was used to cover culture flasks and was solidified with 102mM CaCl₂.

On the scaffold prepared as described above, 3T3 fibroblasts were seeded with density 10⁵/25cm². After 24h cells' viability was estimated using trypan blue exclusion test. Culture flasks without polymere served as control group. Viability was defined as of viable cells to all cells ratio. The results were presented as means ± SD. Differences between means were compared using t-Student test. Significance was set at P<0.05.

Assessment of implants containing 3T3 cells on alginate and PAA scaffolds

3T3 fibroblasts and C57B1 mice were used in experiment. PAA and alginate scaffolds were obtained as described above. 32 mice were divided into four groups, 8 mice each. The control group consisted of 6 mice. The first group received 1ml PAA s. c. injection, second 2x10⁶ 3T3 cells in 1ml PAA s. c. injection, third 1ml alginate s. c. injection and fourth 2x10⁶ 3T3 cells in 1ml alginate s. c. injection. The control group did not receive any implant. Injections were performed in femoral area, between skin and muscle, forming a blister of 0.5cm diameter so that the neo-tissue would be easy to find. After 2 months, the animals were sacrificed by CO₂ overdose. In the *in vivo* part of the experiment "Principles of Laboratory animal care" NIH publication No. 80-23, revised 1978, as well as specific national laws were followed.

Staining with hematoxyline and eosin

Histological specimens were made of samples from region where polymere/polymere with cells had been injected. They were stained with hematoxyline and eosin and examined using light microscope equipped with digital camera (Nikon Eclipse microscope and Nikon E5400 camera, Precoptic Co, Warsaw, Poland).

Measurement of cathepsin D activity and antitrypsin level

In order to measure activity of cathepsin D, 1ml of blood was obtained from each animal. Cathepsin activity in serum was presented as its activity compared to 10^{-2} nM trypsin/mg of protein/min. Antitrypsin level was estimated using Eriksson method [5]. It was presented as the quantity of trypsin which activity was inhibited by 1 ml of serum. The results were presented as means \pm SD. Differences between means were compared using t-Student test. Significance was set at $P < 0.05$.

RESULTS

In *in vitro* experiment, 3T3 cells were cultured *in vitro* on PAA and alginate scaffolds. Cell growth was compared to the ones on polypropylene. Fibroblasts viability on PAA was around 25% lower than on alginate (Fig. 1).

Macroscopically visible structures were observed in case of implants consisting of cells and PAA or alginate. Implants consisting of cells and alginate had good mechanical properties. During dissection they adhered to muscle. Their diameters were around 5mm. Implants consisting of cells and PAA were macroscopically different to the ones with alginate. These implants had smaller diameters, less than 2mm. Their mechanical properties were unsatisfactory. They disintegrated during mechanical manipulation. Implant integration with muscle was not observed.

Mechanical properties of alginate-based implants were significantly worse in group of mice that received alginate without cells. They disintegrated during mechanical manipulation and no implant integration with muscle was observed. Neo-tissue nor polymere presence was not observed in animals that received only PAA. In those cases, samples were taken from regions of injection.

In histopathological examination of specimens stained with hematoxyline and eosin cells growth on scaffold was observed in both groups of mice that received alginate or PAA with 3T3. Implants underwent significant remodelling and cells growth within them

was clearly visible (Fig. 2, 3). In implants consisting only of alginate acellular, vitreous blue masses were observed. There was no migration of host cells into the scaffold, there was no sign of biomaterial degradation nor implant remodelling in this case.

Increase in both cathepsin D and its inhibitor were found as a reaction to PAA as well as alginate. In case of PAA increase of α_1 -antitrypsin level was lower in comparison to alginate, but the differences were not statistically significant. The increase of cathepsin D activity in PAA group was greater in comparison to alginate in cell treated group ($p=0.01$) as well as in acellular scaffolds group ($p=0.03$) (Fig. 4, 5).

Cathepsin D activity and level of α_1 -antitrypsine in animals that received alginate were also higher than control. The differences, however, were not statistically significant.

DISCUSSION

The aim of this research was to evaluate PAA as scaffold for transplanted cells. 3D matrixes help cells to grow, proliferate and form tissue-like structures. Materials with proper biocompatibility are sought after. In the first part of the experiment, *in vitro* growth of 3T3 fibroblasts on both scaffolds was assessed. 3T3 fibroblasts are often used as an experimental model of cells seeded on a scaffold [6, 7].

In *in vitro* assay cell viability on PAA was lower than on PAA. Reasons of lower 3T3 viability on alginate remain unclear. It may be caused by toxic factors introduced during scaffold preparation. It is possible that modification of PAA to make it water soluble would improve its proprieties. It is worth mentioning that 75% cell viability rate is acceptable and should not impair implant cell survival in host organism.

Reaction to the material is important as well. Scaffold particles should not migrate to other parts of body while host cells should be allowed to migrate into the scaffold. Alginate is a biodegradable polymere used in transplantations of numerous cells: chondrocytes, myocytes, pancreatic islets etc. It can be regarded as a standard in research on new biomaterials [8-12]. In this research, no cell migration into alginate scaffolds was observed – no cells were found in hematoxylin and eosin stained samples. Absence of cell migration into alginate scaffold, which is considered to be a very good biomaterial, has not been described previously.

Mechanical proprieties of PAA were worse than alginate. It appears that modification of the polymere structure by cross-linking would be required as well. It would make PAA more stable scaffold for growing cells in future.

Cathepsin D, a protease located in lysosomes, takes part in extracellular matrix degradation. α_1 -antitrypsin is an inhibitor of many proteases, including cathepsin D. They both may be used as markers of immunological response in degenerative and inflammatory diseases as well as of neoplasms progression. Although cathepsin D is not a specific marker of scaffold degradation *in vivo*, it may be, together with α_1 -antitrypsin level, used to assess systemic response to the implanted material [13-15]. The observed increase of enzyme activity and its inhibitor level may be the evidence of scaffold remodeling process [16]. Every implanted scaffold induces nonspecific inflammatory response. As a part of this reaction, proteases (cathepsin D among them) are released from macrophages.

Increase of its activity indicates increased connective tissue remodeling, for example its increase may be a marker of tissue infiltration [14, 18]. Protease inhibitors protect the constituent parts of the connective tissue matrix. Every transplanted scaffold induces nonspecific inflammatory response. As a part of this reaction, proteases (cathepsin D among them) are released from macrophages. Intensity of this inflammatory response affects cell survival rate [3, 20]. Because of their functions, cathepsin D and α_1 -antitrypsin are useful markers of scaffold remodeling intensity.

Statistically significant increase in cathepsin D activity was observed in animals that received PAA or PAA with cells in comparison to control group. In animals that received alginate or alginate with cells this effect was less apparent. Increase in cathepsin D activity may be the evidence of intense PAA scaffold remodeling, which may be a positive effect as the physiological function of implants is connected with their proper remodeling *in vivo* [7].

Increase of α_1 -antitrypsin levels in groups that received PAA implants was lower in comparison to alginate. Because α_1 -antitrypsin is an inhibitor of many proteases, this may suggest that alginate scaffold induced greater increase of serum enzymatic activity. Differences in enzyme activity between PAA and alginate groups were not statistically significant, significance was obtained only in comparison to control group. The lack of statistically significant differences in enzymatic response between PAA and a standard

scaffold – alginate may suggest that PAA is a promising candidate for a scaffold material. Immunological response to implanted materials still requires further examination.

α_1 -antitrypsin levels in group that received alginate implants without cells were higher in comparison to alginate with 3T3 fibroblasts. This fact may have several implications: first of all, it suggests that cells have a modulating influence on scaffold remodeling, secondly, that implant cells regulate the intensity of enzymatic response, finally, that allogenic cells suspended in scaffold does not induce an intense immunological response [8, 17, 19]. These presumptions, however, require further verification.

There are few reports relating PAA as a biomaterial in tissue engineering or regenerative medicine [3]. The aim of this experiment was to compare proprieties of PAA with alginate. The results suggest that PAA is a promising biomaterial for scaffolds used in cell culture and transplantation. This polymer requires modifications in order to improve its mechanical proprieties and its water solubility.

CONCLUSION

1. Enzymatic response was lower after injection of biomaterial with cells, in this case neo-tissue was formed in implantation site. Cells had an influence on proper biomaterial degradation *in vivo*.

2. PAA scaffold may induce less intensive inflammatory response than alginate. PAA still requires certain modifications.

LITERATURA

- [1] Kim B. S., Baez C. E., Atala A.: Biomaterials for tiussue engieneering. World J. Urol., (2000),18, 2-9.
- [2] Polaczek J., Pielichowski J., Dziki E.: Synteza poli(kwasu asparaginowego) jako materiału stosowanego w inżynierii biomedycznej. Inżynieria Biomateriałów, (2003), 23, 21.
- [3] Cai K., Yao K., Hou X., Wang Y., Hou Y., Yang Z., Li X., Xie H.: Improvement of the

- functions of osteoblasts seeded on modified poly(D,L-lactic acid) with poly(aspartic acid). *J. Biomed. Mater. Res.*, (2002), 62, 283-291.
- [4] Polaczek J., Pielichowski J., Pielichowski K., Tylek E., Dziki E.: Nowa metoda syntezy poli(kwasu asparaginowego) w warunkach promieniowania mikrofalowego. *Polimery*, (2005), 50, 11-12.
- [5] Szczeklik E.: *Enzymologia kliniczna*. PZWL Warszawa, 1974;
- [6] Drewa T., Gałązka P., Prokurat A., Wolski Z., Sir J., Wysocka K., Czajkowski R.: Abdominal wall repair using a biodegradable scaffold seeded with cells. *J. Pediatr. Surg.*, (2005), 40, 317-321.
- [7] Drewa T., Sir J., Czajkowski R., Woźniak A.: Scaffold seeded with cells is essential in urothelium regeneration and tissue remodeling in vivo after bladder augmentation using in vitro engineered graft. *Transplant Proc.*, (2006), 38, 133-135.
- [8] Bottino R., Fernandez L. A., Ricordi C., Lehmann R., Tsan M. F., Oliver R., Inverardi L.: Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. *Diabetes*, (1998), 47, 316-323.
- [9] Caldamone A. A., Diamond D.: Long-term results of the endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes. *J. Urol.*, (2001), 165, 2224-2247.
- [10] Diamond D., Caldamone A. A.: Endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes: preliminary results. *J. Urol.*, (1999), 162, 1185-1188.
- [11] Puelacher W.C., Mooney D., Langer R., Upton J., Vacanti J. P., Vacanti C.A.: Design of nasoseptal cartilage replacements synthesized from biodegradable polymers and chondrocytes. *Biomaterials*, (1994), 10, 774-778.
- [12] Risbud M., Ringe J., Bhone R., Sittering M.: In vitro expression of cartilage-specific markers by chondrocytes on a biocompatible hydrogel: implications for engineering cartilage tissue. *Cell Transplant*, (2001), 10, 755-763.
- [13] Wittlin S., Rösel J., Hofmann F., Stover D. R.: Mechanisms and kinetics of cathepsin D activation. *Eur. J. Biochem.*, (1999), 265, 384-393.
- [14] Conus S., Perozzo R., Reinheckel T., Peters C., Scapozza L., Yousefi S., Simon H.U.: Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the

- resolution of inflammation. J. Exp. Med. 2008 Feb 25 [Epub ahead of print].
- [15] Hausmann M., Obermeier F., Schreiter K., Spottl T., Falk W., Schölmerich J., Herfarth H., Saftig P., Rogler G.: Cathepsin D is up-regulated in inflammatory bowel disease macrophages. Clin. Exp. Immunol., (2004), 136, 157-167.
- [16] Vashishta A., Saraswat Ohri S., Vetvickova J., Fusek M., Ulrichova J., Vetvicka V.: Procathepsin D secreted by HaCaT keratinocyte cells - A novel regulator of keratinocyte growth. Eur. J. Cell Biol., (2007), 86, 303-313.
- [17] de Vos P., Smedema I., van Goor H., Moes H., van Zanten J., Netters S., de Leij L. F., de Haan A. de Haan B. J.: Association between macrophage activation and function of micro-encapsulated rat islets. Diabetologia, (2003), 46, 666-673.
- [18] Lavezzi A., Mantovani M., Della Berta L. G., Matturri L.: Cell kinetics of human nasal septal chondrocytes in vitro: importance for cartilage grafting in otolaryngology. J. Otolaryngol., (2002), 31, 366-370.

Adres autorów

Zakład Inżynierii Tkankowej
Katedra Biologii Medycznej
Collegium Medicum
Uniwersytet Mikołaja Kopernika
ul. Kałowicza 24, 85-090 Bydgoszcz
Tel: +48-52-585-3737; Fax: +48-52-585-3742
E-mail: tomaszdrewa@wp.pl